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(71) Applicants *(for all designated States except US)*: MASSACHUSETTS INSTITUTE OF TECHNOLOGY [US/US]; 77 Massachusetts Avenue, Cambridge, MA 02139 (US). FORSYTH DENTAL CENTER [US/US]; 140 Fenway, Boston, MA 02115 (US). OHIO STATE UNIVERSITY [US/US]; 1960 Kenny Road, Columbus, OH 43210 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : STILLS, Harold, P., Jr. [US/US]; 1720 Riverhill Road, Columbus, OH 43221 (US). FOX, James, G. [US/US]; 349 Littleton Road, Harvard, MA 01451 (US). PASTER, Bruce, J. [US/US]; 983 South Street, Portsmouth, NH 03801 (US). DEWHIRST, Floyd, E. [US/US]; 140 Medford Street, Arlington, MA 02174 (US).

(74) Agents: BROOK, David, E. et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US).

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(57) Abstract

The present invention relates to the discovery of a novel intracellular bacterium in hamsters with transmissible proliferative ileitis. This novel intracellular bacterium has been isolated in purified form and, on the basis of phylogenetic analysis, has been determined to possess 95 % similarity to *Chlamydia psittaci* and 98 % similarity to *Chlamydia trachomatis*. Thus, it has been discovered that the "*Campylobacter*-like organisms" long implicated in proliferative ileitis in hamsters, and likely in proliferative bowel diseases in other species as well, are not members of *Campylobacter* genus at all, but rather appear to be a novel species of *Chlamydia*.

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STRAIN OF CHLAMYDIA

Description

Background of the Invention

Proliferative ileitis (transmissible ileal hyperplasia; wet-tail) is a disease of hamsters characterized by mucosal hyperplasia and by a pyogranulomaeous inflammation of the distal ileum. The hyperplastic phase of the disease precedes the inflammatory phase and is characterized by replacement of the normal epithelium with an immature pseudostratified epithelium with a high mitotic index. As the disease progresses, the villi elongate and become leaflike, and the proliferating crypts penetrate into the underlying supporting tissues and Peyer's patches. Secondary bacterial invasion may result in abscess formation, peritonitis, and ileal rupture. Bacteria, viral agents, and neoplasia have all been implicated as possible etiologies for the disease.

Light microscopic studies have revealed a gram-negative, slightly curved bacillus within the hyperplastic ileal cells of hamsters with proliferative ileitis. An antibody reactive with this bacillus is produced by hamsters naturally infected with the disease and by those inoculated with homogenized ileal lesions. Ultrastructurally, the organism resembles members of the genus *Campylobacter*. For this reason, the appellation "Campylobacterlike" has been used in the literature to describe these mysterious bacteria, widely believed to be yet-unidentified members of the genus *Campylobacter*.

Microbiologic studies have repeatedly associated *Campylobacter jejuni* with both naturally occurring and experimentally induced proliferative ileitis in hamsters.

However, oral inoculation of hamsters with pure cultures of C. jejuni has been uniformly unsuccessful in reproducing the disease. Surgical inoculation of C. jejuni into the ileum has produced diarrhea and enterocolitis, but not proliferative lesions. Campylobacter hyointestinalis has also been isolated from hamsters, but attempts to reproduce proliferative ileitis by oral inoculation of pure cultures of C. hyointestinalis, as well as Campylobacter mucosalis and Campylobacter coli, have also been unsuccessful.

Proliferative bowel diseases, or enteropathies, characterized by intraepithelial Campylobacter-like bacteria are well-known in swine as well as hamsters, and have also been reported in ferrets, sheep, rats, guinea pigs, dogs, a foal, a blue fox, Moluccan rusa deer, and rabbits.

The etiology and pathogenesis of these proliferative bowel diseases have been poorly understood. Efforts to identify the intracellular bacteria in swine and hamsters have been unsuccessful, and few studies have been conducted in other species. The consistent location and morphology of the organisms and the similar general characteristics of the lesions in a wide variety of host species have tended to implicate the intracellular bacteria as the etiologic agent(s).

It has been known that the disease could be produced in pigs and hamsters by inoculation with preparations of homogenized intestinal tissue containing intracellular Campylobacter-like bacteria, and that these preparations were no longer pathogenic after passage through filters which retained bacteria. Successful treatment of enteritis

with antibiotics in rabbits and ferrets also supported the contention that bacteria had at least a contributing etiologic role. However, it has not been possible to obtain cultural isolates of the intraepithelial bacteria or to reproduce the natural disease in gnotobiotic hosts.

Porcine proliferative enteritis (swine proliferative enteritis; porcine necrotic enteritis; porcine hemorrhagic enteropathy; porcine intestinal adenomatosis) is a disease which occurs naturally in swine and has been linked for some time with the intracellular presence of Campylobacter-like organisms. It is pathologically comparable to hamster proliferative ileitis (Jonas et al., J. Am. Vet. Med. Assoc., 147:1102-1108), and weanling hamsters have proven to be susceptible to the agent of porcine proliferative enteritis by cross-species transmission (McOrist et al., Veterinary Microbiology, 15:293-302 (1987)). Porcine proliferative enteritis occurs in pigs of weaning age or older but is primarily observed in feeder pigs (14 to 15 kg) and in gilts (bred), sows, and boars. The disease is worldwide in distribution, having been reported in the United States, United Kingdom, Sweden, Australia, Denmark, and Taiwan.

Gross lesions are characterized by thickening of the wall of the ileum primarily, but also of the jejunum, cecum, and colon. There are reticulation of the serosa, hyperemia and edema of the mesentery, and enlarged ileocecal lymph nodes. The affected intestinal segment may contain variable amounts of fibrin, necrotic material, and blood in the lumen. The underlying mucosa has prominent mucosal folds and a granular appearance and is hyperemic.

Occasionally, there are diffuse areas of mucosal necrosis and prominent thickening of the tunica muscularis.

Microscopically, there are diffuse proliferation of crypt epithelium, elongation of crypts, and flattening of the villous surface. Variable numbers of macrophages, eosinophils, and neutrophils are present in the lamina propria. There is superficial to full-thickness mucosal necrosis, accompanied by exudation of fibrin, leukocytes, and erythrocytes into the intestinal lumen.

Under electron microscopy, Campylobacter-like organisms (CLO) found in conjunction with either hamster or swine enteritis are free within immature epithelial cell cytoplasm, are 2 to 3 μm long and 0.2 to 0.3 μm in diameter, and have a trilaminar, undulating cell wall. The organism apparently penetrates immature crypt epithelial cells preferentially, by passing through the microvillous border of these cells. In more severely affected tissues, there is confluent multifocal to diffuse degeneration of the superficial epithelium 5 characterized by rounding, vacuolation, and sloughing of epithelial cells.

While results of immunofluorescence studies have indicated that the organism within the ileal enterocytes differs from known Campylobacter species, most speculation has centered around the proposition that the organism is an as yet uncultured Campylobacter species. (McOrist et al., FEBS Microbiology Letters, 69:189-194 (1990)). Despite a tremendous amount of research interest and activity in the field, the identity of this mysterious intraepithelial organism has remained shrouded in speculation, due to an inability to obtain cultural

isolates and/or reproduce the natural disease in gnotobiotic hosts.

Summary of the Invention

The present invention relates to the discovery of a novel intracellular bacterium in hamsters with transmissible proliferative ileitis. This novel intracellular bacterium has been isolated in purified form and, on the basis of phylogenetic analysis, has been determined to possess 95% similarity to Chlamydia psittaci and 98% similarity to Chlamydia trachomatis.

Thus, it has been discovered that the "Campylobacter-like organisms" long implicated in proliferative ileitis in hamsters, and likely in proliferative bowel diseases in other species as well, are not members of Campylobacter genus at all, but rather appear to be a novel species of Chlamydia. This purified and isolated bacterium, hereinafter referred to as Chlamydia sp. strain SFPD, was deposited at the American Type Culture Collection (ATCC), Rockville, Maryland, on August 21, 1991, and accorded Accession No. M83313.

The present invention also relates to rapid and effective methods for diagnosis of transmissible proliferative ileitis. As used herein, transmissible proliferative ileitis includes transmissible ileal hyperplasia in hamsters, porcine proliferative enteritis in swine, porcine necrotic enteritis, porcine hemorrhagic enteropathy, and porcine intestinal adenomatosis, as well as other of the so-called "CLO-associated" proliferative bowel diseases in animals.

This invention also relates to methods to identify infected swine, hamsters, ferrets, rabbits, *etc.*, especially those which are symptomatic and shedding the organism in their feces.

Either monoclonal or polyclonal antibodies can be utilized for such diagnostic purposes, in both feces and tissues, and can be directed against either the genus Chlamydia or this novel strain of Chlamydia in particular. Alternatively, DNA probes useful for clinical diagnosis of the aforementioned proliferative bowel diseases, and characterized by a high degree of specificity and selectivity for the organism of the present invention, can be advantageously employed now that the identity and phylogenetic structure of the organism have been deduced.

This invention further relates to methods of transferring passive immunity to susceptible animals using antibodies that are reactive with Chlamydia sp. strain SFPD, and to vaccines prepared from the novel Chlamydia sp. strain SFPD.

Detailed Description of the Invention

The intracellular organism of the present invention was isolated from ileal epithelial cells of a hamster with transmissible proliferative ileitis (TPI). The procedure followed is set forth in considerable detail in Example 1, but will be briefly summarized here.

The ileal epithelial cells were isolated by hyaluronidase digestion and gradient centrifugation. The ileal epithelial cell suspension was then lysed with 0.2% Triton X-100 and the intracellular organism collected by

centrifugation following filtration of the lysed suspension through a 0.65 μm filter.

The organism was isolated following inoculation of 70-80% confluent Intestine 407 human embryonic intestinal cell (ATCC CCL 6) monolayers. Growth of the organism was first detected at day 8 post-inoculation and was maintained in cell culture by repeated passage.

As further described in Example 1, typical lesions of proliferative ileitis were successfully reproduced in hamsters inoculated per os with the infected cell-free filtrate and 0.65-μm-pore-size filtrate, while littermates given the uninfected control cellfree filtrate and 0.2-μm-pore-size filtrate remained free of disease.

The organism then was reisolated from one of the 0.65-μm-pore-size filtrate-inoculated hamsters which developed proliferative ileitis. The organism was not recovered from hamsters inoculated with uninfected control cell filtrate or the 0.2-μm-pore-size filtrate.

Thus, the novel strain of Chlamydia appears to fulfill the criteria to be considered an etiologic agent of transmissible proliferative ileitis. The 0.2μm pore-size filtrate did not reproduce the disease, suggesting that the causative organism is larger than a virus. Extensive transmission electron microscopic examinations also failed to detect any viral particles or other bacteria within the infected cells. The nearly identical ultrastructural morphology of this novel strain of Chlamydia within the infected cells utilized in the examples and infected hamster ileal epithelium supports the role of the bacterium in disease pathogenesis.

Pursuant to identification of the causative agent as a novel strain of Chlamydia, modification of the disclosed isolation procedures to utilize antibiotics to which Chlamydia are typically resistant would be advantageous. Utilization of vancomycin, amphotericin B, and members of the aminoglycoside group of antibiotics (gentamicin, neomycin, streptomycin, etc.) in cell culture isolation effectively suppresses contaminating bacterial organisms while permitting Chlamydia growth.

While a substantial body of "evidence" had evolved over the past several years suggesting that the intracellular organism observed within the intestinal cells in hamster and swine proliferative enteritis cases was a Campylobacter species, heretofore, Chlamydia had never been implicated. In fact, respected researchers in the field had gone so far as to publish journal articles dismissing Chlamydia as a potential causative agent (McOrist et al., Research in Veterinary Science, 46:27-33 (1989)). This failure to implicate, or even consider, Chlamydia as an agent is believed to stem, in part, from the atypical behavior exhibited by the novel strain of Chlamydia described herein.

The two known species of genus Chlamydia, C. trachomatis and C. psittaci, are known to have an affinity for epithelial cells of mucous membranes, and attachment and penetration appear to involve a heat-labile surface component on the infectious elementary bodies and a trypsin-sensitive receptor on the host cells.

The elementary bodies enter by a phagocytic process, creating a phagosome or inclusion in which the microcolony develops. While the n vel Chlamydia of the present

invention does form inclusion bodies in culture, it tends not to be vacuolated in the host. Thus, because this intracellular organism was typically found free in the cytoplasm, it was thought to be a Campylobacter rather than a Chlamydia.

16S rRNA was isolated and sequenced as described in detail in Example 2. Briefly, for 16S RNA sequencing, the novel bacterium was isolated from infected cell culture and media supernate by centrifugation at 800 x g to remove cell debris, followed by isolation of the organism at 12,000 x g. The complete 16S rRNA sequence was determined for the organism using a modified Sanger dideoxy chain termination method, wherein primers complementary to conserved regions were elongated with reverse transcriptase.

Based on phylogenetic analysis, the novel strain of intracellular bacterium was shown to be related to Chlamydia, as discussed in Example 2. Based on this analysis, the organism isolated in conjunction with the present invention is believed to be a new strain, and possibly a new species, of Chlamydia.

Effective diagnosis of transmissible proliferative ileitis in hamsters, porcine proliferative enteritis, and other proliferative bowel diseases has been hindered by the time required to culture the causative organism from fecal specimens and by the difficulty in distinguishing the novel organism of the present invention from the various species of Campylobacter which also inhabit the intestines of affected species. As a result of the work presented herein, development of diagnostic tools promoting rapid and accurate assays for the presence of

this novel strain of Chlamydia, Chlamydia sp. strain SFPD, in biological samples taken from hamsters, swine, ferrets, rabbits, and other species susceptible to proliferative bowel diseases is now possible.

For example, as described in Example 1, polyclonal antibodies, reactive with Chlamydia sp. strain SFPD can be used in diagnostic immunoassys, such as ELISAs, RIAs, to detect the presence of Chlamydia sp. strain SFPD in biological samples which include fecal material and tissue.

Likewise, monoclonal antibodies can be generated using the Chlamydia sp. strain SFPD, isolated by the methods described herein, and standard techniques well known to those skilled in the art. These monoclonal antibodies can be used in diagnostic assays as described above.

Similarly, the isolated Chlamydia sp. strain SFPD can be used as an antigen in an immunoassay, such as the ELISA described in Example 1, to detect antibodies to Chlamydia sp. strain SFPD found in the serum of animals infected with the bacterium.

Isolation of this novel strain of Chlamydia makes possible a determination of the nucleotide sequences of its 16S rRNA molecule, which in turn, makes possible the development of nucleic acid probes to aid in its detection.

Nucleic acid probes are a sensitive and rapid alternative to culture methods for the detection of pathogens, particularly in the case of fastidious or noncultivatable organisms. These probes can be either DNA or RNA. DNA probes, for example, are now in common usage

and can be custom-designed in terms of their sizes and specificities for a variety of prospective applications. The sizes of these probes can range from entire plasmids (kilobases in size) down to simple 10- to 15-base synthesized oligonucleotides.

DNA probes can be tailored to bind to other DNA, ribosomal RNA (rRNA), or messenger RNA (mRNA). A given probe will bind to specific nucleotide sequences. A radioactive, enzymatic, or organic label bound to the probe allows it to be detected.

DNA probes specific for rRNA sequences have been used successfully in the detection of various pathogens, and various diagnostic kits based on DNA probes are now available for use in clinical labs. The presence of Chlamydia sp. strain SFPD in biological samples can be detected using these nucleic acid probes. For example, a sample of fecal material from an animal can be hybridized to a DNA probe which is complementary to all, or a portion, of the 16S rRNA sequence (SEQ. ID. NO: 1)

Five DNA probes (SEQ. ID. NOS: 3, 5, 7, 9 and 11) which hybridize to target sequences of Chlamydia sp. strain SFPD have been developed as described in detail in Example 3.

Additionally, antibodies reactive with Chlamydia sp. strain SFPD can be used to transfer passive immunity to animals susceptible to the bacterium. These antibodies, either polyclonal or monoclonal, can be formulated for therapeutic use as a pharmaceutical composition comprising appropriate carriers, excipients, and other pharmaceutically acceptable ingredients, as is known to those skilled in the art of pharmacology. Like other

proteinaceous materials, the antibody preparation is formulated as a sterile, non-pyrogenic composition for parenteral administration. However, any pharmaceutically acceptable route and method of administration of antibody for passive transfer of immunity can be used, such as those described in a standard reference text in this field (e.g., Remington's Pharmaceutical Sciences).

The isolated and purified bacterium Chlamydia sp. strain SFPD can itself be used in a vaccine formulation to be administered to susceptible animals. The Chlamydia sp. strain SFPD can be attenuated so that, although the bacterium remains infectious so that it retains its ability to elicit an immune response in the animal, the attenuated bacterium does not cause significant disease in the host animal. Thus, the administration of the vaccine formulation induces the host animal to produce antibodies reactive against subsequent challenge with Chlamydia sp. strain SFPD, thereby neutralizing the infecting bacterium and preventing significant disease.

Attenuation can be achieved by multiple passaging of the organism and selecting for a weakened strain. Attenuation can also be achieved by mutation of the bacterium using techniques known to those skilled in the art.

Alternately, the vaccine formulation can be comprised of killed, not just attenuated, Chlamydia sp. strain SFPD. Killing can be achieved by heat treatment or lethal doses of chemicals, such as formalin. In either case, the bacterium comprising the vaccine formulation is capable of eliciting an immune response in the host animal without significant diseas .

The vaccine will be formulated for therapeutic use as a pharmaceutical composition comprising appropriate carriers, excipients, and other pharmaceutically acceptable ingredients, as described above and as known to those skilled in the art of pharmacology. The vaccine formulation may also contain additional materials, such as adjuvants, to increase the immunogenicity of the vaccine. Serum titers of antibodies elicited in response to immunization with these vaccine formulations can be determined by use of the diagnostic assays described above.

The present invention will now be illustrated by the following examples, which further and more specifically illustrate the invention.

EXAMPLE I: Isolation of an intracellular bacterium from hamsters with proliferative ileitis and reproduction of the disease with a pure culture

Isolation procedures

The aseptically removed ilea of three hamsters with experimentally induced proliferative ileitis (21 days post-inoculation of infected ileal homogenate) provided the source material. The ilea were cut into 2.5-cm sections, opened longitudinally, and repeatedly washed with Hank's balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+} and 1 mM EDTA (HBSS-EDTA) to remove the intestinal contents. The mucus was removed by incubating the ileal sections in 20% (wt/vol) N-acetyl-cysteine (Sigma Chemical Co., St. Louis, Mo.) for 45 min at 37°C with agitation. The supernatant was decanted, and the sections were washed

twice in HBSS-EDTA and then three times in HBSS. The sections were then transferred to 20 ml of hyaluronidase (1 mg/ml) in HBSS with 5% (wt/vol) bovine serum albumin (BSA) and incubated at 37°C for 2 hr with agitation to free the epithelial cells. The resulting solutions were successively filtered through a sterilized glass bead-filled syringe, a 105-μm-pore-size polypropylene mesh, and a 52-μm-pore-size nylon mesh (Spectra/Mesh; Spectrum Medical Industries, Inc., Los Angeles, Calif.) to remove the larger tissue fragments.

The crude cell suspensions were centrifuged (400 x g for 10 min) and resuspended in HBSS twice. Following a final centrifugation, the cell pellets were resuspended in 50 ml of Leibovitz L-15 media (Sigma) with 10% fetal calf serum, 100 μg of gentamicin sulfate (Sigma) per ml, and 2/5 μg of amphotericin B (Sigma) per ml and incubated overnight at 4°C with agitation.

The cells were separated by centrifugation (400 x g for 10 min) and washed three times in HBSS-EDTA with 5% BSA and resuspended in a minimal volume of HBSS-EDTA with 5% BSA. The cell suspensions were then layered onto preformed (15 min at 15,000 x g and at 4°C) 30% Percoll (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.) gradients in HBSS-EDTA with 5% BSA and centrifuged at 400 x g for 15 min. The epithelial cell bands (density 1.035 g/ml) were suspended in HBSS with 5% BSA and washed four times in HBSS with 5% BSA followed by centrifugation (400 x g, 10 min).

The cell pellets were resuspended in HBSS with 5% BSA and 0.2% Triton X-100 and incubated at 37°C for 30 min for agitation to effect cell lysis. The suspensions were then

centrifuged (400 x g, 10 min) to remove cell fragments, and the supernatants were filtered through stacked 20-μm-pore-size nylon (Micron Separations, Inc., Westboro, Mass.) and 0.65-μm-pore-size nitrocellulose filters (Whatman Co., Clifton, N.J.). The filtrates were centrifuged at 10,000 x g for 15 min at 4°C, and the resulting bacterial pellets were washed once in HBSS, recentrifuged, and resuspended in HBSS.

Microbiologic procedures

Three Trypticase soy agar-5% sheep blood plates were inoculated with 250 μl of each bacterial suspension. All plates were incubated at 37°C. One plate was incubated at ambient atmosphere, one was incubated in an atmosphere consisting of approximately 6% O_2, 7% CO_2, 7% H_2, and 80% N_2 obtained as described previously, and the remaining plate was incubated anaerobically. The aerobic and microaerobic plates were examined daily for growth, while the anaerobic plate was examined every 3 days for growth. All plates were held for a minimum of 12 days prior to being discarded.

Cell culture isolation procedures

All cell lines were obtained from the American Type Culture Collection (Rockville, Md.). Culture media, heat-inactivated fetal calf serum, media supplements, and additives were obtained from Sigma.

Intestine 407 human embryonic intestinal cells (ATCC CCL 6) and GPC-16 guinea pig colonic adenocarcinoma cells (ATCC CCL 242) were grown in Dulbecco's modified Eagle's medium with 4,500 mg of glucose per liter, 110 mg of

sodium pyruvate per liter, 584 mg of L-glutamine per liter, and 10% fetal calf serum (DME). HaK Syrian hamster kidney cells were grown in Eagle's minimum essential media with Hank's salts and 10% fetal calf serum. Vero African green monkey kidney cells were grown in medium 199 containing Hank's salts and 10% fetal calf serum. All cell lines were plated onto 75-cm^2 culture flasks (Corning Glass Works, Corning, N.Y.) in the respective media and incubated at 37°C in a 5% CO_2 atmosphere until approximately 50% confluent. Each cell line was then inoculated with 500 μm of each isolated bacterial suspension and incubated at 37°C in a 6% CO_2, 6% O_2, 88% N_2 atmosphere. Cell cultures were split 2:1 by scraping on days 4 and 9 of incubation and examined daily by phase microscopy (Olympus INT; Olympus Corp., Lake Success, N.Y.) for evidence of bacterial growth. All cell cultures were maintained for a minimum of 14 days before negatives were discarded.

Cell cultures with intracellular bacterial growth were continued in cell culture by scraping and splitting through four doublings. The cell culture supernatant was used to infect additional flasks of the same cell line to expand the culture. Infected cells were frozen at -80°C in the respective media with 10% dimethyl sulfoxide for reference and later use.

Inocula preparation

Four inocula were prepared for the infection studies. The inoculum for the control group (group A) was prepared from two 75-cm^2 flasks of confluent uninfected Intestine 407 cells. The cells were mechanically separated from the

flask by scraping, concussion, and vortexing. Triton X-100 was then added to the cell media suspension to a concentration of 0.2% (vol/vol), and the suspension was incubated at 37°C with agitation for 20 min. The suspension was then centrifuged (400 x g, 20 min), and the supernatant was filtered through a 20-μm-pore-size nylon filter (Micron Separations, Inc.). The resulting filtrate was centrifuged at 11,000 x g for 20 min at 4°C, and the resulting pellet was resuspended in 10 ml of DME.

The infected inocula were prepared in a similar manner except that a total of six 75-cm^2 flasks were processed. Following the filtration through a 20-μm-pore-size nylon filter, the infected inocula were divided into three portions. The group B inoculum was processed identically to the group A control inoculum. The group C inoculum was filtered through a 0.65-μm-pore size cellulose filter (Whatman) prior to final centrifugation, and the group D inoculum was similarly filtered through a 0.2-μm-pore-size cellulose filter (Whatman) prior to final centrifugation. All inocula were held at 4°C after preparation and were utilized within 3 hr of preparation.

Animals

Four adult Syrian hamsters (Hsd;SYR) with 17-day-old litters were purchased from a commercial supplier whose colony had no previous history of proliferative ileitis. The weanling hamsters were separated from their dams, sexed, weighed, ear tagged, and placed into group cages by gender. Post-inoculation, hamsters were housed in conventional polycarbonate rodent cages and provided a commercial rodent diet (RMH 3000; Agway, Inc., Syracuse,

N.Y.) and water ad libitum. Each hamster was weighed at 3- to 5-day intervals by placing the animal in a clean pretared container.

Animal inoculation

The weanling hamsters were divided into four groups with a conscious attempt to equalize the sex and weight distribution among the groups. Each hamster was given 7.5 mg of cimetidine (Tagamet; SK&F Lab Co., Cidra, Puerto Rico) intraperitoneally 30 min prior to inoculation to induce temporary achlorhydria. Following oral inoculation by gavage, each hamster was given 0.2 mg of dexamethasone (Azium; Schering Corp., Kenilworth, N.J.) intramuscularly.

Control group A hamsters (5 male and 3 female) were inoculated with 1 ml of a cell-free filtrate from uninfected Intestine 407 cells. Group B hamsters (5 male and 3 female) were inoculated with 1 ml of a cell-free filtrate from infected Intestine 407 cells. Groups C and D hamsters (4 male and 4 female each) were inoculated with 0.65-μm- and 0.2-μm-pore-size filtrates from infected Intestine 407 cells.

Necropsy and tissue processing

Hamsters were killed by an intraperitoneal injection of 25 mg of sodium pentobarbital at day 28 postinoculation. A terminal blood sample of 2 ml was drawn from the right ventricle for serum titer determination by enzyme-linked immunosorbent essay (ELISA), and the ileum was aseptically removed. A small section of the ileum was fixed in McDowell-Trump fixative for light and electron microscopy. The remainder of the ileum was either frozen

(-80°C) for later use or further processed for organism reisolation.

Sections for light microscopy were routinely processed, embedded in paraffin, cut at 4 μm, and stained with hematoxylin and eosin and with Warthin-Starr silver stains for microscopic evaluation. Light microscopic sections were coded and evaluated without knowledge of experimental group assignment.

Reisolation procedures

Reisolation procedures were identical to those utilized in the initial isolation with the exception that only Intestine 407 cells were inoculated for organism reisolation. The ilea of 3 hamsters from group C (0.65-μm-pore-size filtrate inoculated) and 2 hamsters from group A (control cell inoculated) were independently processed for reisolation of the organism.

Transmission electron microscopy

Hamster ileal sections fixed in McDowell-Trump fixative were washed twice in 0.122 M Millonig's buffer, immersed in 1.33% osmium tetroxide in 0.122 M Millonig's buffer, dehydrated in graded ethanol solutions, cleared in propylene oxide, and embedded in Epon 812. Alkaline toluidine-blue-stained thick sections were examined for selection of thin sections. Thin sections were mounted on 300-mesh copper grids, stained with lead citrate-uranyl acetate, and examined with a Philips 300 electron microscope.

Infected Intestine 407 cells (passage 13) for electron microscopy were removed from the culture flask by

scraping, pelleted by centrifugation (400 x g, 10 min), and fixed in 2.5% glutaraldehyde in Millonig's buffer. The cells were then processed as described above except for the necessity of pelleting the cells by centrifugation between steps.

ELISA technique

The terminal serum samples, day 28 postinfection, were evaluated for serum titer to the organism by ELISA. A crude whole-cell preparation was used as the antigen in the ELISA procedures. Supernatant from infected Intestine 407 cell cultures (passages 25 to 32) was centrifuged at 400 x g for 10 min to remove large particulates and cells. The supernatant was then centrifuged at 12,000 x g for 15 min at 4°C to pellet the bacteria. The pellet was washed once with phosphate-buffered saline (PBS; 0.01 M, pH 7.4, with 0.02% Na_3N), recentrifuged, resuspended in PBS to a density corresponding to a McFarland standard of 2, and refrigerated until used. Campylobacter jejuni (ATCC 29428), isolated from brucella broth, was similarly processed as a control antigen.

Ninety-six-well assay plates (E.I.A. Microtiter Plates; Flow Laboratories McLean, Va.) were coated with a 1:64 dilution of both the isolated bacterial antigen and C. jejuni at 100 111 per well and dried overnight at 37°C. Wells were incubated with 100 111 of absolute methanol containing 0.3% H_2O_2 for 30 min at 37°C to inactivate endogenous peroxidase activity. The plates were then washed twice in ELISA wash buffer (0.15 M NaCl, 0.1 M Tris-HCl, 0.02% Tween 20 (pH 7.4)) and incubated for 1 hr at room temperature with 200 μl of PBS containing 0.20%

(vol/vol) Tween 20 and 2.5% (wt/vol) nonfat powdered milk per well. Plates were washed twice with ELISA wash, and 100 µl of test sera diluted from 1:100 to 1:12,800 in PBS was applied for 1 hr. at room temperature. After being washed 3 times, plates were incubated for 1 hr. at room temperature with 100 µl of peroxidase-labeled goat anti-hamster immunoglobulin G (IgG) diluted 1:2,500 in ELISA wash per well. Following an additional 3 washes, bound antibody was detected by the addition of 100 µl of 2,2'-azinobis (3-ethylbenzthiazoline sulfonic acid) substrate (Sigma) for 15 min., and the A_{405} was determined with an automated ELISA reader (Titertek Multiscan; Flow Laboratories). All assays were performed in duplicate and were repeated if values differed by greater than 10%. Absorbance values greater than 0.2 absorbance units above that of the control (C. jejuni) were considered positive. Pooled sera from female hamsters from a closed colony without a history of proliferative ileitis were included as a negative control with all tests.

Polyclonal antibody production

The bacterial isolate was purified from Intestine 407 cell culture (passage 15 and 16) for antibody production. Culture supernatant was centrifuged at 400 x g for 10 min to remove cell debris, and the resulting supernatant was filtered through a 0.65-µm-pore-size cellulose acetate filter (Whatman). The filtrate was then centrifuged (12,000 x g for 15 min at 4°C), and the pelleted organisms were washed once in PBS, recentrifuged, and resuspended in 10 ml of PBS containing 4% formalin. Following overnight incubation at 4°C, the organisms were again isolated by

centrifugation, washed once in Dulbecco's PBS, recentrifuged, and resuspended in Dulbecco's PBS to a concentration approximating a McFarland standard of 0.5. The organism suspension was then emulsified with an equal volume of Fruend's complete adjuvant (Sigma Chemical Co.).

Two male rabbits were utilized for antibody production. Each rabbit was injected with a total of 0.5 ml of the adjuvant-organism mixture divided into 10 sites intradermally on the back. A booster injection of 1.0 ml of the organism in Dulbecco's PBS was given subcutaneously at 40 days postinjection, and sera were collected 7 days later.

The IgG fraction of the rabbit antisera was purified by precipitation with ammonium sulfate (33% final concentration) followed by ion exchange chromatography. The resulting IgG fractions were then reprecipitated with ammonium sulfate, isolated by centrifugation, and desalted on a Sephadex G25 column (Pharmacia LKB Biotechnology, Inc.) equilibrated and eluted with PBS.

Immunohistochemistry

Fixed ileal sections embedded in paraffin and cut to 4 μm were utilized for immunohistochemistry. Sections were routinely deparaffinized in xylene and rehydrated through decreasingly concentrated ethanol solutions. The sections were then incubated for 1 hr in 0.03% H_2O_2 in absolute methanol to remove endogenous peroxidase activity. Following washing in PBS, the sections were incubated for 1 hr in a blocking solution of 0.2% (vol/vol) Tween 20 and 2.5% (wt/vol) nonfat dry milk in PBS. Following an

additional wash, the rabbit anti-organism antibody was applied at a dilution of 1:100 in PBS, and the slides were incubated overnight at 4°C in a humidified chamber. The slides were then washed 4 times in ELISA wash and incubated for 3 hrs with affinity-purified peroxidase-labeled goat anti-rabbit IgG (Jackson Immunoresearch) diluted 1:500 in ELISA wash. The sections were then washed 4 times in ELISA wash, and bound antibody was detected with a commercial peroxidase detection system for immunohistochemistry (HistoMark Black; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) by following the manufacturer's directions. Controls to ensure specificity included both infected and uninfected Intestine 407 cells, preimmunization rabbit sera, and organism-adsorbed immune rabbit antibody.

Results

Isolation and propagation of the organism

Inoculation of cell cultures with two of the bacterial suspensions resulted in heavy bacterial contamination and discarding of the cell cultures after 18 hr of incubation. Cell cultures inoculated with the remaining bacterial suspension appeared to be free of bacterial contamination, incubation was continued, and the flasks were split (2:1) on day 4. Intracellular organisms were first observed in both the Intestine 407 and GPC-16 cell lines by phase microscopy on day 8 following inoculation. Clusters of minute organisms within the cells were readily identified by their rapid motility. The clust rs of organisms were xtremely variable in siz ,

from barely discernible within the cell cytoplasm at a 4000 x magnification to large clusters completely filling the distended cells. Occasional cells with small clusters of organisms were observed in mitosis with the cell cluster apparently transferring to one of the daughter cells. No evidence of intracellular bacteria was observed in either the HaK or Vero cell lines.

The organism continued to multiply in the original cell cultures (with additional 2:1 splittings on days 14, 18, and 20) through day 19 postinoculation (Intestine 407) and day 21 postinoculation (GPC-16), when the cultures were contaminated. The organism was readily and continually (more than 40 passages) propagated by infecting confluent Intestine 407 cell cultures with the supernatant from infected cultures (1 ml of infected supernatant per 25-cm^2 surface area). During the first 5 passages in cell culture, intracellular organisms could be detected by phase microscopy within 72 hr of infection, with the interval between infection and microscopic detection decreasing in later passages. Cell culture monolayer viability decreased from 10 days (passage 1) to 6 days by passage 5. Following the initial 5 passages in cell culture, organisms were detected by phase microscopy at 24 hr post cell culture infection, and monolayer destruction was evident in 4 to 6 days following infection. No changes in bacterial morphology were evident following up to 40 passages in cell culture. Growth of the organism appeared to be substantially greater on the Intestine 407 cell line and passage 12 of this line was utilized for inoculum preparation.

Necropsy results

The gastrointestinal tract of all control-inoculated (group A) and 0.2-μm-pore-size filtrate-inoculated (group D) hamsters appeared grossly normal at necropsy. No evidence of epithelial cell proliferation or intracellular bacteria was present on light microscopic examination of hematoxylin- and eosin-stained or Warthin-Starry-stained ileal sections from hamsters in either of these groups.

In 4 (2 male and 2 female) of the 8 group B hamsters (cell-free infected inoculate) and 4 (3 male and 1 female) of the 8 group C hamsters (0.65-μm-pore-size filtrate), the ileum was grossly thickened. Ileal wall thicknesses varied from 2 to 5 mm in these cases. Ileal wall necrosis and abscessation with secondary fibrinopurulent peritonitis was present in 3 of these hamsters (1 from group B and 2 from group C). Light microscopic examination of ileal sections from hamsters with grossly evident ileal thickening revealed immature and disorganized villus epithelial cells with mitotic figures extending from the crypt region up the entire length of the villus. Numerous small curved bacteria were visible in the apical cytoplasm of the epithelial cells in silver-stained ileal sections of all hamsters with grossly evident ileal thickening. A mixed inflammatory cell infiltrate of neutrophils and macrophages was present in the lamina propria of all hamsters with gross lesions. Mild focal epithelial cell proliferation and intracellular bacteria were present in an additional 2 (1 from group B and 1 from group C) of the 8 hamsters that appeared normal at gross necropsy.

Reisolation results

Three of the 5 bacterial suspensions inoculated into Intestine 407 cell cultures resulted in heavy bacterial contamination at 18 hr of incubation, and these cultures were discarded. The remaining 2 cell cultures (1 group A and 1 group C) remained free of bacterial contamination through day 7 of incubation when intracellular organisms were detected by phase microscopy in the group C (0.65-μm-pore size filtrate) inoculated cell culture. Intracellular bacteria were not detected in the group A (control) inoculated cell culture through 14 days of incubation.

Microbiologic results

Of the 8 bacterial suspensions, 3 from the original isolation and 5 from reisolation, only those resulting in contamination of the cell cultures produced any growth on Trypticase soy agar-5% sheep blood plates. Pseudomonas aeruginosa, 1 to 8 colonies per 250 μl, was isolated from all 5 contamination suspensions. No other isolates were detected by any of the incubation conditions.

Transmission electron microscopy results

Numerous intracellular bacteria were observed within the cytoplasm of ileal epithelial cells from hamsters inoculated with the 0.65-μm-pore-size filtrate (group C). The bacteria were free within the epithelial cell cytoplasm. Macrophages with intracellular bacteria both free in the cytoplasm and in phagosomes were present adjacent to epithelial cells. The bacteria were typically slightly curved on longitudinal sections and measured 1.4

to 1.6 μm in length by 0.25 to 0.38 μm in width. The bacterial cell wall appeared trilaminar and irregular. No flagella were observed.

Examination of infected Intestine 407 cells revealed the presence of bacteria morphologically identical to those seen in the ileal cells of infected hamsters. The bacteria in the Intestine 407 cells measured 1.4 to 1.7 μm in length by 0.26 to 0.34 μm with a similar trilaminar irregular cell wall. A majority of the bacteria in the Intestine 407 cells were free within the cell cytoplasm, although some were membrane bound within vacuoles.

ELISA results

Serum titers of hamsters from group A (control inoculated) ranged from <1:100 to 1:1,600, with a median titer of 1:400. The serum titers from the group D (0.2-μm-pore size filtrate inoculated) hamsters ranged from <1:100 to 1:800, with a median titer of 1:400. Serum titers of hamsters from group C (0.65-μm-pore size filtrate inoculated) ranged from 1:1,600 to >1:12,800, with a median titer of 1:6,400, while the titers of the group B hamsters ranged from 1:1,600 to >1:12,800, with a median of 1:3,200. The serum titers of the group B and group C hamsters were significantly ($P < 0.01$, Mann-Whitney test) higher than the titers of either group A or group D hamsters. The highest titers, ranging from 1:6,400 to >1:12,800, were recorded from hamsters with grossly evident ileal thickening at necropsy. The pooled negative control sera consistently titered at <1:100.

Immunohistochemistry results

Organisms labeled with the polyclonal rabbit anti-organism antibody were present in the ileal sections from all 10 hamsters with histologic evidence of proliferative ileitis. Organisms were primarily confined to the apical portions of ileal epithelial cells, with few organisms being present in the lamina propria or within the lumen. The distribution of organisms corresponded to the distribution of silver-stained organisms seen on the Warthin-Starry-stained sections from the same hamster. No stained organisms were present within the ileal epithelial cells from any of the 8 group A or 8 group D hamsters.

Organisms specifically labeled with the polyclonal rabbit anti-organism antibody were also present in the infected Intestine 407 cells. No reactivity was present with uninfected Intestine 407 cells, and reactivity was completely eliminated by adsorption of the rabbit antisera with formalin-fixed organisms.

EXAMPLE 2: 16S rRNA sequencing of the intracellular bacterium isolated in Example 1 and its identification as a novel strain of Chlamydia

16S rRNA sequencing

The complete 16S rRNA sequence was determined for the organism isolated in Example 1 using a modified Sanger dideoxy chain termination method wherein primers complementary to conserved regions were elongated with reverse transcriptase. The complete sequence (SEQ ID NO:1) is shown in Table 1

TABLE 1

UUCUGAGAAUUUGAUCUUGGUUCAGAUUGAACGCUGGCGGCGUGGAU
GAGGCAnGCAAGUCGAACG----GAACGAUUGCUUCGGUGAUUGUUU----
AGUGGCGGAAGGGUUAGUnAUACAUAGAUnAUUUGUCCUUAACUUGGG
AAUAACGGUUGGAAACGGCCGCUAAUACCGAAUGUGGCGUAGAUUAG-G
CaUCUAAAUUACGUUAAAGAAGGGGA-UCUUCGGA-CCUUUCGGUUAAG
GGAGAGUCUAUGUGAUAUCAGCUAGUUGGUGGGGUAAAGGCCUACCA
AGGCUAUGACGUCUAGGCGGAUUGAGAGAUUGGCCGCCAACACUGGGA
CUGAGACACUGCCCAGACUCCUACGGGAGGCUGCAGUCGAGAAUCUUU
CGCAAUGGACGGAAGUCUGACGAAGCGACGCCGCGUGUGUGAUGAAGG
CUC-UAGGGUUGUAAAGCACUUUCGCUUGGGAAUAAGA-GAAGACGGUU
AAUACCCGUUGGAUUUGAGCGUACCAGGUAAAGAAGCACCGGCUnACU
CCGUGCCAGCAGCUGCGGUnAUACGGAGGGUGCUaGCGUUAAUCGGAU
UUaUUGGGCGUAAAGGGCGUGUaGGCGGAAAGGUAAGUUAGUUGUCAA
AUCUCGGGGCUCnACCCCgAnUCGGCAUCUnAAACUnUUUUUCUAGAGG
GUAGAUGGAGAAAAGGGaAUUUCaCGUGUAGCGGUGAAAUGCGUAGAUA
UGUGGAAGAACACCAGUGGCGAAGGCGCUUUUUnUnAUUUAUACCUGACG
CUAAGGCGCGAAAGCAAGGGGAGCAAACAGGAUUaGAUACCCUnGUAG
UCCUUGCCGUaAACGAUGCAUACUUGAUGUGGAUGGU--CUCAAC-CCC
AUCCGUGUCGGAGCUAACGCGUUAAGUAUGCCGCCUGAGGAGUACACU
CGCAAGGGUgAAACUCAAAAGAAUUGACGGGGGCCCGCACAAGCAGUGG
AGCAUGUgGUUUaAUUCGAUGcaACGCGAAGGACCUUACCUGGGUUUGA
CAUGCA-UAUGACCGCGGCAGAAAUGUCGUUU--UCCGCAAGGA---CAU
AUGCA-CAGGUGCUGCAUGGCUGUCGUCAGCUCGUGCCGUGAGGUGUU
GGGUUAAGUCCCGCAACGAGCGCAACCCUUAUCGUUAGUUGCCAGCAC-
UUAGG--GUGGGAACUCUAACGAGACUGCCUGGGUUnACCAG-GAGGAAG
nnnAngAUGACGUCAAGUCAGCAUGGCCCUUAUGCCCAGGGCGACACACG
UGCUACAAUGGCCAGUACAGAAGGUAGCAAGAUCGCGAGAUGGAGCAA
AUCCUC-AAAGCUGGCCCCAGUUCGGAUUGUAGUCUGCAACUCGACUAC
AUGAAGUCGGAAUUGCUAGUAAUGGCGUGUCAGCCAUAACGCCGUGAA
UACGUUCCCGGGCCUUGUACACACCGCCCGUCACAUCAUGGGAGUUnG
UUUUACCUUAAGUCGUUGACUCAACCCGCAAGGGGGAGAGGCGCCCAA
GGUnAGGCUGAUG

In the foregoing sequence, A represents the base adenine, C represents the base cytosine, G represents the base guanine, U represents the base uracil, lower-case letters (a, c, g, etc.) are believed to be their upper-case equivalent although this is not known with certainty, n indicates that the identity of the nucleotide is unknown, and (-) represents a gap in the sequence as aligned with the 16S rRNA sequence of E. coli.

Similarity matrix

Based on phylogenetic analysis, the novel strain of intracellular bacterium was related to Chlamydia, as shown in Table 2.

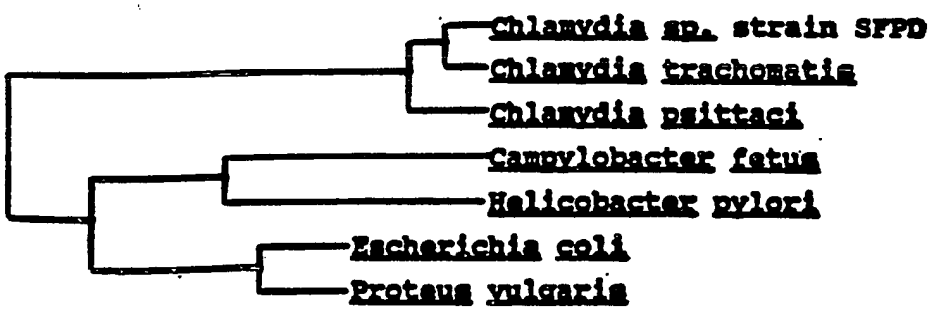
TABLE 2

C_s	C_t	C_p	C_r	H_p	H_e	P_v	
----	98.4	95.6	72.6	72.9	74.1	73.7	Chlamydia sp. strain SFPD
1.65	----	95.2	72.2	72.9	73.9	73.8	Chlamydia trachomatis
4.5	4.9	----	72.5	73.0	74.1	73.4	Chlamydia psittaci
34.1	34.7	34.2	----	85.4	76.9	76.0	Campylobacter fetus
33.7	33.7	33.5	16.2	----	75.2	75.0	Helicobacter pylori
31.7	32.0	31.8	27.6	30.1	----	92.9	Escherichia coli
32.4	32.7	32.8	29.0	30.4	7.5	----	Proteus vulgaris

The numbers above the diagonal represent percent similarity. The numbers below the diagonal are percent differences corrected for multiple base changes by the method of Jukes and Cantor (1969).

The relationship among the above species can be summarized as shown in Table 3.

TABLE 3



Example 3: Development of DNA Probes

1. Determination of oligonucleotide signatures

Signatures are defined as those segments of the RNA sequence which are unique to any given species or group of phylogenetically-related organisms. Signatures can be found by examination of aligned sequences, noting regions in the desired species or higher phylogenetic group that contain differences at sites conserved in other species. Once such a sequence is found, large numbers of reference sequences are searched (by computer) to verify the uniqueness of the sequence. The sequence of Chlamydia sp. strain SFPD was compared with over 350 sequences of other bacteria. These signature regions become the target site for DNA probes.

2. Probe Synthesis

Once the desired oligonucleotide signatures are determined for probe development, DNA that is complementary to the sequence of the signature can be synthesized using techniques well known to those skilled in the art. For example, an ABI DNA Synthesizer can be used to synthesize the oligonucleotides which serve as probes.

3. Probe Labeling

$^{32}PO_4$-ATP can be used as a substrate by T4 polynucleotide kinase for 5' end-labeling of DNA oligonucleotides. Briefly, the procedure is as follows. 0.5 ug of oligomer (probe) and 200 uCi radiolabeled ATP are combined and dried under vacuum. The dried pellet is

dissolved in 0.05 ml of kinase buffer containing 11.5 U of Polynucleotide kinase (Pharmacia). This mixture is incubated at 37°C for 30 min. Ammonium acetate and EDTA are then added to stop the reaction. The reaction mixture (0.5 ml) is gravity loaded onto C-8 Bond-Elute columns (Analytichem. Intern.) and is washed 3X with 1 ml of 50 mM ammonium acetate and then once with 1 ml of 5 mM ammonium acetate.

4. Probe specificity to RNA and DNA

Probes can be hybridized with rRNA samples that were used for sequencing reactions. Once these probes have proven to be successful using purified RNA, the probes can be validated using pure cultures of target organisms (Strain SFPD) and related organisms (e.g., other strains of chlamydia). Probes can target 16S rRNA or 16S rRNA regions of the genome.

The hybridization procedure has been adapted from Schleicher & Schuell (Keene, N.H.) and is as follows: Approximately 2-4ug rRNA is denatured in a solution of 6.15 M formaldehyde in 10X SSC (NaCl/Na Citrate buffer) at a temperature of 65°C for 15 min. The denatured rRNA is transferred to a prewashed nitrocellulose (NC) filter in a Slot Blot apparatus (Schleicher and Schuell). This apparatus is a modified dot blot which allows for rapid, high resolution hybridization of nucleic acids. The denatured RNA is baked onto the filter at 80°C for 1h under vacuum. The bake filter is placed in a seal-a-meal plastic bag and hybridization solution containing formamide, Salmon testis DNA, Denhardts solution, EDTA and SDS is added to the bag. After 20 min incubation at the

hybridization temperature (Th), the solution is removed and replaced with a fresh hybridization solution containing approximately 1 x 10^7 cpm of labeled probe. Hybridization of the labeled probe to the rRNA on the filter is carried out at the Th for 18h. After 3 washes at RT with 0.8X SSC (low stringency), the NC is washed 2 times at the Th with 0.8X SSC for 30 min each. Most of the wash is removed, leaving the filter still wet. The filter still in the baggie is read on a Betascope 603 (Betagen, Waltham, MA) which allows for the rapid imaging (i.e., in less than 1 hour) and quantification of radioactive samples.

Probe Sequences
Probe position: 63
Probe length: 24 -mer
Target sequence: (SEQ ID NO: 2)
Probe sequence: (SEQ ID NO: 3)

CGAACG-----GAACGAUUGCUUCGGUGA	Chlamydia sp. strain SFPD
.....G.A....U.....CA.	C. trachomatis L2/434
.....GAGUA-UUAAGAGAGC.U.C.NU	Camp. fetus ss fetus
.....AUGAA-GCU-UC.AGC.U.C.AG	Helicobater pylori
.....GUAACAG-GA.GAAGC.U.C.UC	Escherichia coli
...G..GUAACAG-GAGAAAGC.U.C.UU	Proteus vulgaris

In the example above, the 16S rRNA sequence of strain SFPD is compared with the sequences of 6 other microorganisms. Thus, there are 5 base differences between Chlamydia sp. strain SFPD and C. trachomatis and 6

base differences between Chlamydia sp. strain SFPD and C. psittaci.

Probe position: 167
Probe length: 24 -mer
Target sequence: (SEQ ID NO: 4)
Probe sequence: (SEQ ID NO: 5)

CGCUAAUACCGAAUGUGGCGUAGA	Chlamydia sp. strain SFPD
.....AUAU	C. trachomatis L2/434
.....UA.-.U	Chlamydia psittaci 6BC
U.....UCC..ACUC.U.-CU	Camp. fetus ss fetus
GAU.....AG..UCUC.U----	Helicobacter pylori
A.....C..AAC.UC----	Escherichia coli
G.....C...AC.UC----	Proteus vulgaris

Probe position: 205
Probe length: 24 -mer
Target sequence: (SEQ ID NO: 6)
Probe sequence: (SEQ ID NO: 7)

CUAAAUUACGUUAAAGAAGGGGA-U	Chlamydia sp. strain SFPD
.CG.G.A.....	C. trachomatis L2/434
.....AC.UA.....	Chlamydia psittaci 6BC
-GUU.AGU..GG....U-----.-	Camp. fetus ss fetus
--------G.GG.....-----.-	Helicobacter pylori
--------GACC.....G.....C	Escherichia coli
--------GACC.....C.....U..	Proteus vulgaris

Probe position: 455
Probe length: 24 -mer
Target sequence: (SEQ ID NO: 8)
Probe sequence: (SEQ ID NO: 9)

GAAGACGGUUAAUACCCGUUGGAU	Chlamydia sp. strain SFPD
....G.....C.....	C. trachomatis L2/434
..GAUU..C.....U..AA.C...	Chlamydia psittaci 6BC
----------C	Camp. fetus ss fetus
----------	Helicobacter pylori
..GU.AA.....UU.GCUCA	Escherichia coli
UG.U.AA.....UU.GUC.A	Proteus vulgaris

Probe position: 591
Probe length: 24 -mer
Target sequence: (SEQ ID NO: 10)
Probe sequence: (SEQ ID NO: 11)

AGGUAAGUUAGUUGUCAAAUCUCG	Chlamydia sp. strain SFPD
.....GA.....	C. trachomatis L2/434
...A.....A...U.....U.	Chlamydia psittaci 6BC
UAUC....CUU....G.....AA	Camp. fetus ss fetus
UA..C...C..G...G.....CUA	Helicobacter pylori
U.U.....C..A...G.....C.C	Escherichia coli
-AU.....C..A...G...G.C.C	Proteus vulgaris

Equivalents

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically her in. Such quival nts are intended to be enc mpassed in the scope of the following claims.

CLAIMS

1. A substantially pure culture of Chlamydia sp. strain SFPD.

2. The substantially pure culture of Claim 1 comprising Chlamydia sp. strain SFPD accorded ATCC Accession No.M83313.

3. A substantially pure culture of one or more of Chlamydia accorded ATCC Accession No. M83313, taxonomic equivalents thereof, and mutants or variants thereof.

4. The purified and isolated organism of Claim 3 Chlamydia sp. strain SFPD.

5. The purified and isolated organism of Claim 4 containing a 16S ribosomal RNA sequence encompassed by SEQ ID NO:1.

6. A method for isolating Chlamydia sp. strain SFPD from the intestinal epithelial cells of an animal which comprises isolating said epithelial cells by hyaluronidase digestion.

7. The method as described in claim 6 wherein said animal is a hamster, pig, rabbit or ferret.

8. A method for isolating Chlamydia sp. strain SFPD which comprises propagating the organism in a reduced- xygen envir nment.

9. A method for isolating Chlamydia sp. strain SFPD which comprises propagating the Chlamydia sp. strain SFPD on Intestine 407 human embryonic intestinal cells or GPC-16 guinea pig colonic adenocarcinoma cells.

10. A method for reproducing transmissible proliferative ileitis in animals which comprises inoculating an animal with the substantially pure culture of Claim 1.

11. A method for generating polyclonal antibodies reactive with Chlamydia sp. strain SFPD which comprises injecting a host animal with the Chlamydia sp. strain SFPD or cellular components thereof.

12. A polyclonal antibody reactive with Chlamydia sp. strain SFDP produced by the method of Claim 11.

13. A method for generating monoclonal antibodies reactive with Chlamydia sp. strain SFPD which comprises injecting a host animal with Chlamydia sp. strain SFPD or cellular components thereof.

14. A monoclonal antibody reactive with Chlamydia sp. strain SFPD produced by the method of Claim 13.

15. A method for detecting Chlamydia sp. strain SFPD in a biological material sample from an animal, comprising:

a) obtaining a biological sample from an animal;

b) contacting the sample with an antibody reactive with Chlamydia sp. strain SFPD under conditions whereby the antibody reacts with the Chlamydia sp. strain SFPD and

c) detecting antibody-bound sample wherein antibody bound to the sample is an indication of the presence of Chlamydia sp. strain SFPD.

16. The method of Claim 15 wherein the biological sample is tissue or fecal material.

17. The method of Claim 15 wherein the antibodies are labeled with a label selected from the group consisting fluorescein, rhodamine, and peroxidase.

18. The method of Claim 15 wherein the biological material sample is derived from hamsters, swine, rabbits, or ferrets.

19. The method of Claim 15 wherein the antibodies are polyclonal antibodies produced by the method of Claim 11.

20. The method of Claim 15 wherein the antibodies are monoclonal antibodies produced by the method of Claim 13.

21. A method for diagnosing transmissible proliferative ileitis in an animal by detecting antibody reactive with Chlamydia sp. strain SFPD present in the serum of an animal, comprising:

a) obtaining a serum sample from the animal;

b) contacting the serum sample with purified and isolated Chlamydia sp. strain SFPD under conditions whereby the antibody present in the serum sample reacts with the Chlamydia sp. SFPD; and

c) detecting serum antibody bound to the Chlamydia sp. strain SFPD wherein antibody bound to the organism is an indication of transmissible proliferative ileitis.

22. The method of Claim 21 wherein the purified and isolated Chlamydia sp. strain SFPD is isolated according to the methods of Claims 6, 8 or 9.

23. A nucleic acid probe for the detection of Chlamydia sp. strain SFPD comprising a nucleic acid which hybridizes to all or a part of the sequence represented by SEQ ID NO:1.

24. The nucleic acid probe of Claim 23 wherein the nucleic acid is DNA.

25. The nucleic acid probe of Claim 23 as represented by SEQ ID NO: 3.

26. The nucleic acid probe of Claim 23 as represented by SEQ ID NO: 5.

27. The nucleic acid probe of Claim 23 as represented by SEQ ID NO: 7.

28. The nucleic acid probe of Claim 23 as represented by SEQ ID NO: 9.

29. The nucleic acid probe of Claim 23 as represented by SEQ ID NO: 11.

30. A method of detecting Chlamydia sp. strain SFPD present in a biological sample in an animal, comprising:

a) obtaining a biological sample from an animal;

b) treating the sample in a manner that renders 16S ribosomal RNA present in the sample available for hybridization with a complementary nucleic acid sequence thereby producing a treated sample;

c) contacting the treated sample with at least one probe which is a nucleic acid sequence which hybridizes with all or a portion of a nucleic acid sequence represented by SEQ ID NO:1; and

d) detecting the hybridization of 16S ribosomal RNA from the sample with the nucleic acid probe, wherein hybridization is an indication of the presence of Chlamydia sp. strain SFPD.

31. A method of detecting Chlamydia sp. strain SFPD present in a biological sample in an animal, comprising:

a) obtaining a biological sample from an animal;

b) treating the sample in a manner that renders 16S ribosomal RNA regions of the genome present in the sample available for hybridization with a complementary nucleic acid sequence thereby producing a treated sample;

c) contacting the treated sample with at least one probe which is a nucleic acid sequence which hybridizes with all or a portion of a nucleic acid sequence represented by SEQ ID NO:1; and

d) detecting the hybridization of 16S ribosomal RNA regions of the genome from the sample with the nucleic acid probe, wherein hybridization is an indication of the presence of Chlamydia sp. strain SFPD.

32. The method of Claim 30 wherein the nucleic acid probe is selected from the group consisting of: SEQ ID NO.: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11.

33. The method of Claim 30 or 31 wherein the biological sample is tissue or fecal material.

34. A method of passively transferring immunity against Chlamydia sp. strain SFPD to an animal comprising administering to the animal an antibody reactive with Chlamydia sp. strain SFPD.

35. The method of Claim 33 wherein the antibody is a polyclonal antibody produced by the method of Claim 11.

36. The method of Claim 33 wherein the antibody is a monoclonal antibody produced by the method of Claim 13.

37. A vaccine formulation comprising an attenuated Chlamydia sp. strain SFPD in which the Chlamydia sp. strain SFPD is infectious without causing significant disease in the animal host to be vaccinated.

38. A vaccine formulation comprising killed Chlamydia sp. strain SFPD in which the killed Chlamydia sp. strain SFPD is infectious without causing significant disease in the animal host to be vaccinated.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/07103

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N1/20; C07K15/00; C12P21/08; G01N33/569
G01N33/577; C12Q1/68; A61K39/40; A61K39/118

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12R

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X,P	BIOLOGICAL ABSTRACTS vol. 42 , 1991, Philadelphia, PA, US; abstract no. 17821, STILLS H ET AL. 'ANEW CHLAYDIA-SP STRAIN SFPD ISOLATED FROM TRANSMISSIBLE PROLIFERATIVE ILEITIS IN HAMSTERS' see abstract & MICROB ECOL HEALTH DIS vol. 4, 1991, page S99 --- -/--	1

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
01 DECEMBER 1992	18. 12. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT FFICE	REMPP G.L.E.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X,P	BIOLOGICAL ABSTRACTS vol. 42 , 1991, Philadelphia, PA, US; abstract no. 17820, FOX J G ET AL. 'LOCALIZATION OF CHLAMYDIA-SP STRAIN SFPD IN CLO ASSOCIATED PROLIFERATIVE INTESTINAL TISSUE OF ANIMALS BY FA MONOCLONAL ANTIBODY AND IN-SITU DNA HYBRIDIZATION' see abstract & MICROB ECOL HEALTH DIS vol. 4, 1991, page S98	1,13-16, 23,24, 30,31

Y	BIOLOGICAL ABSTRACTS vol. 67, no. 7 , 1979, Philadelphia, PA, US; abstract no. 42121, RONSHOLT T I 'INFECTION IN DANISH CATTLE' page 4181 ; see abstract & ACTA PATHOL MICROBIOL SCAND SECT B MICROBIOL vol. 86, no. 5, 1978, pages 291 - 298	1

Y	INNERE MEDIZIN vol. 14, no. 2, March 1987, pages 45 - 48 W. BORNSCHEIN ET AL. 'CHLAMYDIA-TRACHOMATIS-NACHWEIS BEI ENTZÜNDLICHEN UND ULZERÖSEN ERKRANKUNGEN DES DUODENUMS UND ILEUMS'	1

INTERNATIONAL SEARCH REPORT

I national application No.

PCT/US 92/ 07103

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 10 and 34 are directed to a method of treatment of diagnostic method practised on the human/ animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.